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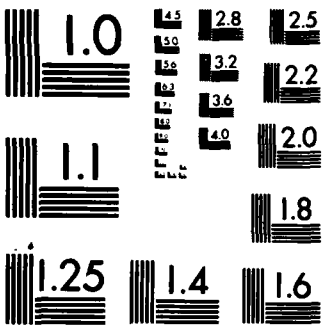

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THE ROLE OF ANTICOAGULATION IN THE MEASUREMENT OF PLATELET VOLUMES

by

C. G. THOMPSON, D. D. DIAZ, P. G. QUINN, M. LAPINS, S. R. KURTZ,

AND

C. R. VALERI

NAVAL BLOOD RESEARCH LABORATORY
BOSTON UNIVERSITY SCHOOL OF MEDICINE
615 ALBANY STREET
BOSTON, MA 02118

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The effect of anticoagulation on platelet size stability was studied using blood collected in seven different anticoagulants and stored at room temperature for up to eight hours. The mean platelet volume (MPV) value was most stable in blood collected in 15% ACD and ACD/Na2EDTA. In blood collected in Na2EDTA, K3EDTA, or 11.9% ACD, there was an increase in MPV in the first two hours after which the MPV's remained stable up to eight hours. Sodium citrate and heparin proved unreliable for the measurement of platelet volume. Platelet counts were stable (<5% variation) in all anticoagulants

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except heparin, which had 16% variation for the eight hours of study. Simultaneously, RBC counts and mean corpuscular volume (MCV) measurements were stable in all seven anticoagulants with sodium citrate producing the most variation. A negative correlation was observed between MCV and pH of the anticoagulated blood. WBC counts showed less than 3% variation in all anticoagulants except sodium citrate and heparin.

Separate experiments demonstrated that electrolyte composition, pH, tonicity, and method of calcium chelation all influenced the stability of the MPV. Of the anticoagulants studied ACD/Na2EDTA appeared to provide the best conditions of anticoagulation for both routine clinical and research laboratory measurement of theMPV. It inhibited platelet activation but left the platelets in their normal discoid shape. Platelets could be removed from the anticoagulant and studied in functional assays for up to eight hours after blood drawing. Both platelet counts and MPV's remained stable in blood collected in ACD/Na2EDTA anticoagulant for up to eight hours at room temperature. In 52 volunteers studied, an inverse correlation ($r = -0.72$, $p < 0.001$) was observed between platelet count and MPV, suggesting that the circulating platelet mass may be a more important indicator of platelet homeostasis than either the platelet count or the mean platelet volume alone.

ABSTRACT

The effect of anticoagulation on platelet size stability was studied using blood collected in seven different anticoagulants and stored at room temperature for up to eight hours. The mean platelet volume (MPV) value was most stable in blood collected in 15% ACD and ACD/Na_2EDTA. In blood collected in Na_2EDTA, K_3EDTA, or 11.9% ACD, there was an increase in MPV in the first two hours after which the MPV's remained stable up to eight hours. Sodium citrate and heparin proved unreliable for the measurement of platelet volume. Platelet counts were stable (< 5% variation) in all anticoagulants except heparin, which had 16% variation for the eight hours of study. Simultaneously, RBC counts and mean corpuscular volume (MCV) measurements were stable in all seven anticoagulants with sodium citrate producing the most variation. A negative correlation was observed between MCV and pH of the anticoagulated blood. WBC counts showed less than 3% variation in all anticoagulants except sodium citrate and heparin.

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52 volunteers studied, an inverse correlation ($r = -0.72$, $p < 0.001$) was observed between platelet count and MPV, suggesting that the circulating platelet mass may be a more important indicator of platelet homeostasis than either the platelet count or the mean platelet volume alone.

Keywords: Platelets, Mean Platelet Volume, Complete Blood Count, Anticoagulants

INTRODUCTION

Mean platelet volumes (MPV) have been studied in the research laboratory for over 20 years now (3, 7-11, 15, 16, 20). Platelets produced during active thrombopoietic stimulation as in immune thrombocytopenic purpura (8) or following chemotherapy (2) appear to be larger than normal. In vitro functional testing of size-separated platelet subpopulations has shown that larger platelets aggregate more readily than smaller ones (19). Post-surgically it has been shown that during wound healing selective utilization of the large platelets occurs (21). Widespread application of the MPV to the clinical evaluation of the platelet, in a manner analogous to the use of the mean corpuscular volume (MCV) for the evaluation of red blood cells, until now has been limited by the expense and difficulty of its measurement (13).

Recent advances in automated blood cell counting and sizing have made both the platelet count and MPV routinely available in most clinical laboratories (3, 18). In attempting to establish reference values for normal subjects in our laboratory it was found that the MPV of a sample varied over time using the standard CBC anticoagulant Na_2EDTA. In order to define the optimum anticoagulation conditions for both routine clinical and research laboratory measurement of the MPV, the effects of seven routine anticoagulants used in our laboratory were studied.

MATERIALS AND METHODS

Effects of Anticoagulants on Platelet Size

The donors were nine healthy volunteers, between the ages of 19 and 28, who worked in our laboratory. At the start of the daytime hematology shift, 100 ml of blood was drawn from a donor and placed into vacutainers (Becton-Dickinson, Rutherford, NJ) containing the anticoagulants to be used. The anticoagulants studied included Na_2EDTA, K_3EDTA, heparin, NaCitrate, ACD (15% acid-citrate-dextrose, NIH Formula A), ACD (11.9%) and ACD/Na_2EDTA. Descriptive data on the formulations of the anticoagulants used are presented in Table 1. Samples were analyzed immediately using a Coulter Counter Model S-Plus (Coulter Electronics, Hialeah, FL). Routine measurements made included hemoglobin, hematocrit, erythrocyte count, MCV, white blood cell count, platelet count and MPV. Follow-up measurements on the samples were performed at 2, 4, 6, and 8 hours. Results were analyzed for average MPV, platelet count, RBC count, MCV and WBC count for the 8 hour measurement period and for trends in variation of the measurement over time. The pH of the anticoagulated samples was measured one hour after blood drawing. The change in osmolality by addition of the anticoagulant was calculated from the known formulations and volumes (Table 1).

The Effects of pH, Osmolality, and Electrolyte Content on MPV

In five experiments platelet rich plasma (PRP) was prepared from blood anticoagulated with 15% iso-osmolar ACD by centrifugation of the blood at 160 g for 10 minutes. Aliquots of the PRP were placed in phosphate-buffered saline of either 270, 285, 305, 315, or 330 mOsm/kg.

MPV's were measured after one hour of incubation. In four separate experiments, similarly prepared PRP was aliquoted into phosphate buffered saline (PBS) whose pH was adjusted to within ± 0.03 pH units of 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, or 7.4. The MPV was measured after one hour. A similar study of the relationship of red blood cell MCV with pH was performed on heparinized whole blood. The effect of sodium versus potassium as an extracellular cation on the measurement of the MPV was evaluated in ACD/Na_2EDTA anticoagulated blood. Three hundred µl of either 0.150 M NaCl, 0.150 M KCl or various ratios of the two solutions were added to the blood and the MPV measured after one hour.

Functional and Morphological Evaluation of ACD/Na_2EDTA Anticoagulated Platelets

Morphology of platelets in PRP prepared from ACD/Na_2EDTA anticoagulated blood was examined by phase microscopy. For functional assessment, platelets were isolated from PRP by centrifugation at 750 g for 15 minutes. Platelets were resuspended to a platelet count of 200,000/µl in a triscitrate bicarbonate buffer, pH 7.4, and recalcified (19). The response to 10 µg/ml of collagen was assessed in an aggregometer.

Statistical Analyses

Statistical analyses were performed using the Student's paired t-test and best linear fit correlation coefficients. All calculations were performed on a T155 desk-top calculator (Texas Instruments, Dallas, TX).

RESULTS

In each of the nine studies, blood was drawn from a normal volunteer and placed in the study anticoagulants. The MPV was measured at regular intervals over eight hours, the length of a routine hematology shift, and the results averaged to obtain a mean MPV for the donor using each anticoagulant. The mean and standard deviation of the MPV's from the nine subjects were determined in this manner (Table 2). There is a considerable range of MPV's among the anticoagulants. The MPV's measured in the ACD anticoagulants were all similar to each other and were consistently lower than the MPV's measured in Na_2EDTA or K_3EDTA (Figure 1). Heparin and NaCitrate anticoagulated blood produced intermediate MPV's. The standard deviations were similar for all of the anticoagulants.

To determine the reproducibility of the MPV measurements, the percent deviation of each individual measurement from that donor's average MPV was computed. From this data, the average measurement variation for each anticoagulant was determined (Table 2). While the least variation was seen with the three different ACD anticoagulated samples, the values for all the anticoagulants were well within the machine limitations of 5% suggested by the manufacturer (5).

To determine if there was a variation in the MPV over time, the individual measurements, as a percent of the donor's average MPV, were averaged at each time point (Table 2). For both ACD (15%) and ACD/Na_2EDTA, the variations in the MPV over time were less than their average measurement variations. For all of the other anticoagulants, variations over time were exhibited which were greater than could be

accounted for by the average measurement variation. For all five of these anticoagulants there was a greater increase in the MPV in the first two hours than would be expected from the calculated measurement variations. In addition, the MPV's as measured in these anticoagulants were all greater than those measured in ACD (15%) or ACD/Na_2EDTA. In Na_2EDTA, K_3EDTA, and ACD (11.9%) after the initial increase in MPV, the values remained stable for the rest of the study period. For heparin and NaCitrate anticoagulated samples a decrease in MPV was noted at the latter measurement times.

In order to assure that the measured differences in MPV were not due to selective loss of platelets in the different anticoagulants, the platelet counts were measured simultaneously (Table 3). Platelet counts in all of the anticoagulants, except heparin, were similar. In the heparinized samples there was a loss of platelets at two and four hours followed by a rise in the platelet count in the latter time periods. Over the same time periods there was an increase in the MPV followed by a decrease at the end of the study period. In some experiments there did appear to be a trend of decreasing platelet counts over time in NaCitrate and the measurement variation was 6.5%. In the other anticoagulants, the average measurement variations were less than 5%. In all of the samples the platelet count at the end of the study period exceeded that at the beginning but no one time period exhibited a change greater than the average measurement variation.

RBC counts, MCV's, and WBC counts were measured on all samples (Table 4). Na_2EDTA, ACD (11.9%), ACD (15%), and ACD/Na_2EDTA all produced similar WBC counts and RBC counts with average measurement errors of 1% for RBC's and 3% for WBC's. No trends in either count were

observed over the time period (data not shown). Heparin anticoagulation proved an inaccurate method for the measurement of WBC counts but not RBC counts. NaCitrate was less accurate in measuring both RBC and WBC counts than the other anticoagulants. While the average measurement error of the MCV was less than 1% for all of the anticoagulants except NaCitrate, the MCV measured in Na_2EDTA, heparin and NaCitrate proved to be consistently lower than in any of the ACD-based anticoagulants.

One major difference between the two groups was pH. Blood anticoagulated in Na_2EDTA, heparin or NaCitrate had a pH of greater than 7.1. Blood anticoagulated in ACD (15%), ACD (11.9%) or ACD/Na_2EDTA had pH's of less than 6.8 (Table 1). To investigate pH as the etiology of the differences in MCV, samples were anticoagulated with heparin and then the pH titrated with the addition of HCl. After one hour the MCV was measured. A negative correlation between the MCV and pH was observed ($r = -0.80$, $p < 0.001$) with a 4.6% decrease in MCV occurring between pH's of 7.4 and 6.7.

In the second part of this study the various influences of pH, electrolyte content, and osmolality on MPV were measured in an attempt to explain the differences among MPV's measured in the different anticoagulants. Table 5 shows the effects of osmolality and pH on the measurement of MPV. Osmolality showed an inverse relationship with MPV ($r = -0.99$, $p < 0.001$), however the magnitude of change in the MPV would be small in the range of osmolality changes induced by the anticoagulants (Table 1). Over the pH range of interest, no significant changes in MPV were noted. Because we observed that K_3EDTA gave consistently higher MPV's than Na_2EDTA, the effect of varying the electrolyte composition of the anticoagulant was examined. Blood was

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anticoagulated in ACD/Na_2EDTA and then a 300 µl aliquot of either 0.150 M NaCl or 0.150 M KCl was added. The sample to which KCl was added had an average 3.3% greater MPV than the sample to which NaCl was added.

Based upon all of these results, the properties of ACD/Na_2EDTA anticoagulated platelets were evaluated further. Morphologically they appeared discoid in shape. Functionally, they aggregated well to collagen, thrombin, arachidonic acid, ristocetin, and ADP. In response to collagen (Figure 2) they demonstrated a characteristic response of lag time, shape change, and aggregation. When the complete blood count of 52 normal volunteers was measured in blood anticoagulated in ACD/Na_2EDTA (Figure 3), a statistically significant inverse correlation ($r = -0.72$, $p < 0.001$) was observed between the MPV and the platelet count.

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DISCUSSION

The standard anticoagulants for CBC's, Na_2EDTA and K_3EDTA, caused an increase over time in the MPV, predominantly in the first two hours, presumably on the basis of a platelet shape change from discoid to spherical (23). This change probably began immediately upon exposure of the blood to EDTA, accounting for the larger MPV's measured in the EDTA anticoagulants. While after two hours the MPV remained stable, such alteration in shape limits further functional assessment of the platelets, should the need arise (6, 23). The *in vitro* induction of the shape change also would inhibit the ability to measure the earliest manifestation of *in vivo* platelet activation, platelet shape change (12). This may be important since partially activated platelets have been shown to be able to circulate (4).

Interestingly, Na_2EDTA and K_3EDTA were not interchangeable. Sodium is predominantly an extracellular cation while potassium is primarily an intracellular cation. K_3EDTA caused greater platelet swelling over time than Na_2EDTA. Experiments with similar concentrations of potassium and sodium in ACD anticoagulated blood showed a 3.3% greater platelet swelling with the addition of KCl than with the addition of the same concentration of NaCl.

Heparin did not inhibit the platelets functionally and *in vitro* activation and reversible aggregation occurred over time, as can be seen by the transitory decrease in platelet count and increase in platelet volume at 2-4 hours.

With the citrate-based anticoagulants (NaCitrate, ACD 11.9%, ACD 15%), decreased platelet stability seemed to occur with increasing pH of the citrate anticoagulant. Platelets themselves were remarkably size stable at pH's 6.7 to 7.4. However, the responsiveness of platelets anticoagulated in citrate has been shown to increase with pH to stimulation in this range (1). NaCitrate proved to be an excellent preservative of PRP but not whole blood (C. B. Thompson, unpublished data), presumably because of partial stimulation which occurred as a result of red blood ADP release (22). Differences among MPV's measured in NaCitrate, ACD (11.9%), ACD (15%) could be accounted for by changes in the final osmolality of the blood, induced by the anticoagulants.

Red blood cell MCV's were shown to vary with pH of the blood, accounting for the differences in MCV's measured among the various anticoagulants. This effect has been noted by other investigators (14, 17). However, no trends in MCV's were noted over time in this study.

The combination of ACD and Na_2EDTA as an anticoagulant was studied because of a report by Aster and Jandl (1) that ACD protected platelets from the deleterious effects of EDTA. This observation was confirmed by our data. Platelets anticoagulated in ACD/Na_2EDTA remained discoid and underwent a normal shape change when removed from the anticoagulant and stimulated. In our study, the addition of Na_2EDTA to ACD improved the workability of the platelets, presumably by more completely inhibiting platelet activation. Washed platelets anticoagulated in ACD alone often were difficult to resuspend and centrifugation occassionally induced partial release. The addition of Na_2EDTA to the ACD anticoagulant largely eliminated these problems. The addition of Na_2EDTA to ACD (11.9%) appears to improve the measurement of MPV as calculated by the

average measurement variation (Table 2), presumably by eliminating the error partial activation can have on the MPV when collected in citrate-based anticoagulants.

In conclusion, ACD/Na_2EDTA proved an ideal anticoagulant for the study of mean platelet volumes. It inhibited platelet activation but left the platelets in their normal discoid shape. Using this anticoagulant a significant inverse correlation between the platelet count and the MPV was demonstrated in normal volunteers, suggesting that the MPV may be as important as the platelet count in determining platelet homeostasis.

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FIGURE 1

Size distribution of platelets in platelet-rich plasma isolated from ACD (15%), Na_2EDTA, and ACD/Na_2EDTA anticoagulated blood and stored at room temperature for four hours.

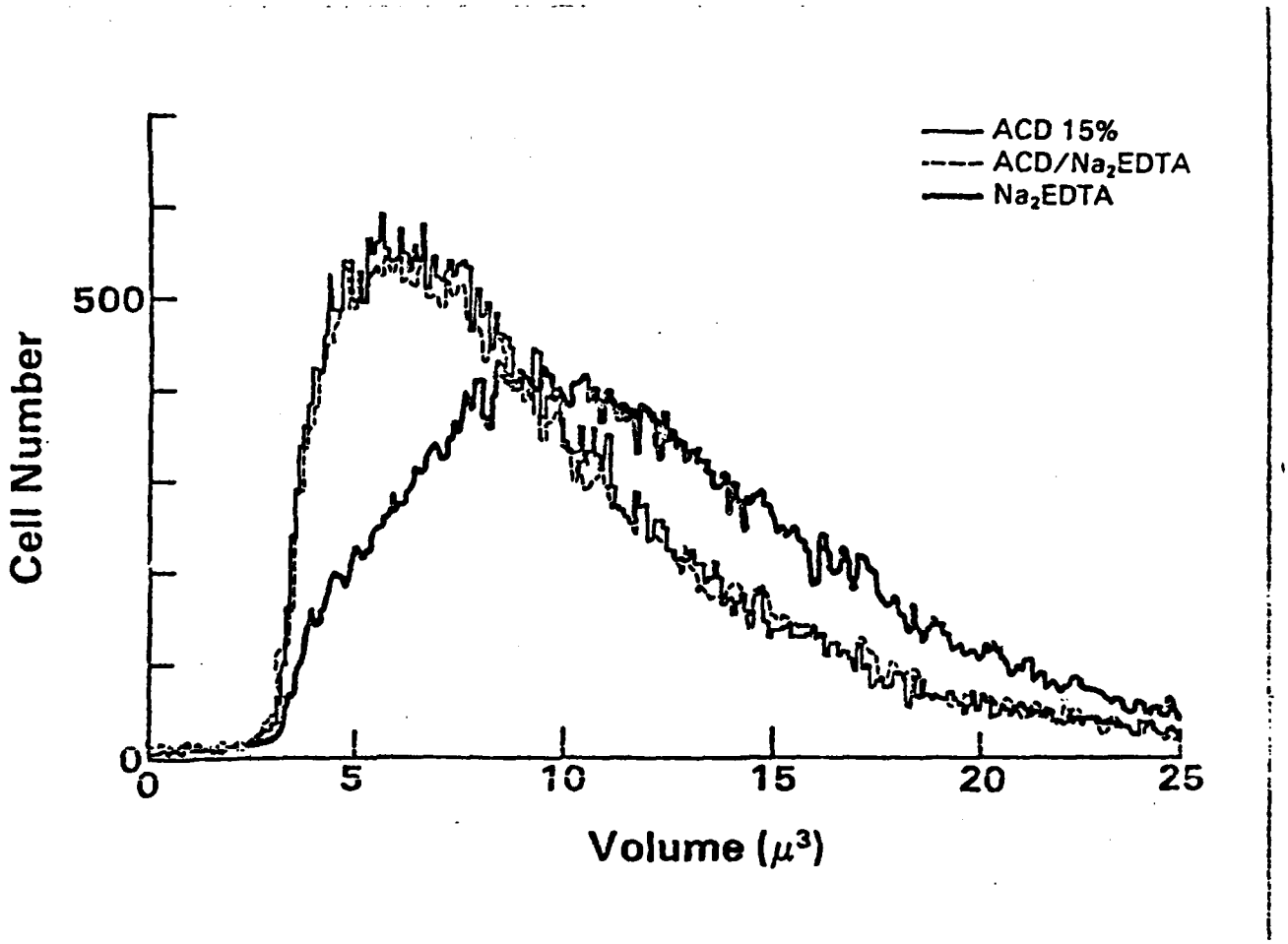

FIGURE 2

Response of platelets isolated from ACD/Na_2EDTA anticoagulated blood to 10 µg/ml collagen stimulation following pH adjustment and recalcification.

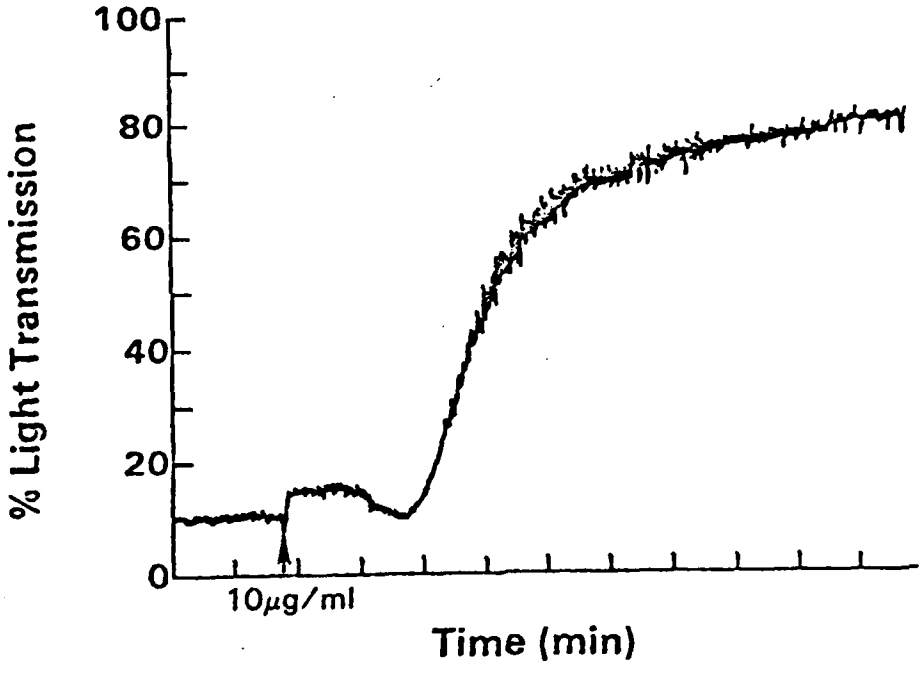

FIGURE 3

The relation between mean platelet volume (μ^3) and platelet count ($\times 10^3/\mu l$) in blood anticoagulated with ACD/Na_2EDTA in 52 healthy individuals.

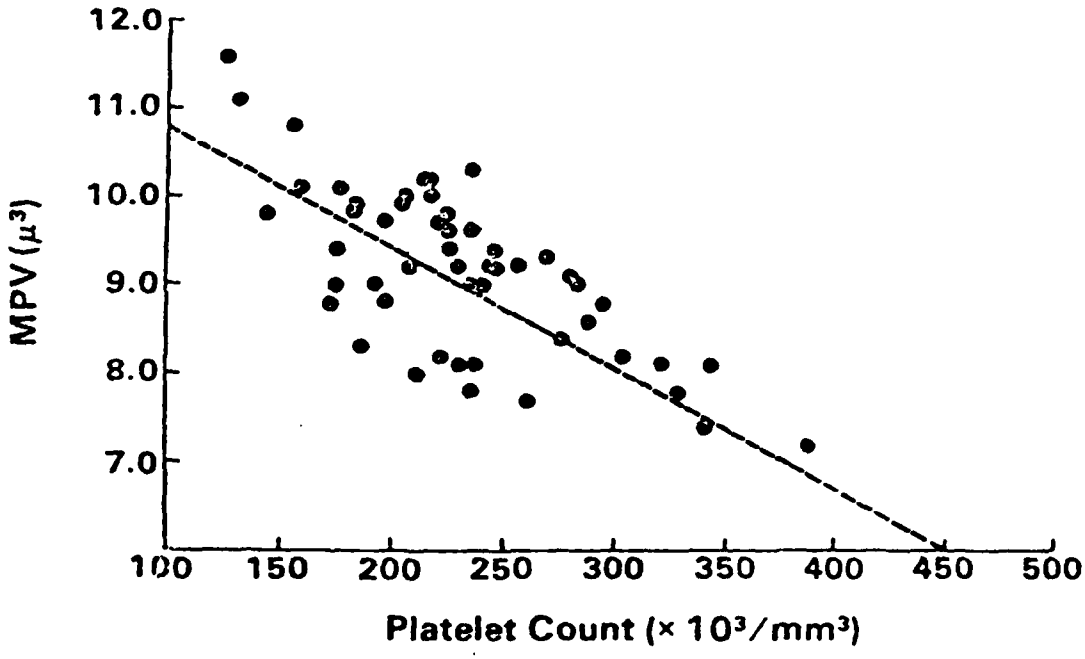

TABLE 1

ANTICOAGULANTS EVALUATED IN THIS STUDY

ANTICOAGULANT	FORMULATION	CALCULATED CHANGE IN OSMOLALITY OF THE BLOOD	FINAL pH OF BLOOD (Mean ± SD, n=3)
Na_2EDTA	15.0 mg/10 ml blood	↑ 15 mOsm/kg	7.14 ± 0.03
K_3EDTA	7.2 mg/5 ml blood	↑16 mOsm/kg	7.28 ± 0.02
Heparin	143 USP units/10 ml blood	--	7.38 ± 0.11
Na Citrate	0.129 M Na citrate 0.5 ml/4.5 ml blood	↓5 mOsm/kg	7.24 ± 0.08
ACD, 11.9%	79.3% USP Formula A in triple distilled water (iso-osmolar) 1.5 ml/8.5 ml blood	0 mOsm/kg	6.75 ± 0.06
ACD, 15%	USP Formula A 1.5 ml/8.5 ml blood	↑12 mOsm/kg	6.64 ± 0.01
ACD (11.9%)/ Na_2EDTA	79.3% USP Formula A in triple distilled water 2% Na_2EDTA in saline 1.5 ml ACD, 0.25 ml Na_2EDTA/ 8.5 ml blood	↑5 mOsm/kg	6.64 ± 0.01

TABLE 2

THE STABILITY OF PLATELET VOLUME IN WHOLE BLOOD COLLECTED IN THE 7 ANTICOAGULANTS AND STORED AT ROOM TEMPERATURE FOR 8 HOURS (Mean ± SD, n=9)

ANTICOAGULANTS	Na_2EDTA	K_3EDTA	HEPARIN	NaCitrate	ACD (11.9%)	ACD (15%)	ACD/ Na_2EDTA
Overall Mean Platelet Volume (μ^3) Over the 8-hour Period	10.8 ± 0.9	11.0 ± 0.8	10.4 ± 1.1	9.6 ± 0.9	9.4 ± 1.0	9.3 ± 0.9	9.3 ± 0.8
Average % Measurement Variation Over 8 Hours	2.7 ± 1.0	3.7 ± 0.7	4.1 ± 1.2	4.4 ± 1.5	2.4 ± 1.6	2.0 ± 0.5	1.8 ± 1.0
Average % of the Overall Volume at Time:							
0 Hr	96.1 ± 1.8	95.9 ± 1.0	97.6 ± 3.1	95.0 ± 4.7	97.9 ± 2.9	99.1 ± 2.3	100.1 ± 2.9
2 Hr	101.6 ± 1.8	102.3 ± 1.0	102.5 ± 2.5	102.8 ± 1.8	100.8 ± 2.2	100.0 ± 1.6	100.3 ± 2.3
4 Hr	101.2 ± 1.2	102.2 ± 0.5	103.1 ± 2.1	100.9 ± 1.8	100.5 ± 1.8	100.2 ± 2.0	100.1 ± 2.3
6 Hr	100.9 ± 1.2	---	99.4 ± 2.5	101.7 ± 3.3	100.7 ± 1.7	100.7 ± 2.1	99.5 ± 1.1
8 Hr	100.5 ± 1.0	---	96.4 ± 3.0	97.7 ± 1.7	100.3 ± 1.8	99.5 ± 1.3	99.7 ± 1.7

TABLE 3

THE STABILITY OF PLATELET COUNT IN WHOLE BLOOD COLLECTED IN THE 7 ANTICOAGULANTS AND STORED AT ROOM TEMPERATURE FOR 8 HOURS (MEAN ± SD, n=9)

ANTICOAGULANTS	Na_2EDTA	K_3EDTA	HEPARIN	NaCITRATE	ACD (11.9%)	ACD (15%)	ACD/ Na_2EDTA
Overall Mean Platelet Count Over the 8-hour Period ($10^3/\mu l$)	224 ± 54	225 ± 59	170 ± 55	222 ± 61	228 ± 57	235 ± 60	232 ± 58
Average % Measurement Variation Over 8-hour	3.1 ± 2.8	3.0 ± 1.8	15.9 ± 7.6	6.5 ± 4.5	2.5 ± 0.6	2.8 ± 1.1	2.8 ± 1.2
Average % of Overall Count at Time:							
0 Hr	97.0 ± 4.6	97.7 ± 1.5	112.8 ± 16.4	102.4 ± 7.6	98.3 ± 1.5	98.4 ± 2.6	98.4 ± 2.4
2 Hr	98.9 ± 3.8	101.7 ± 2.4	84.1 ± 11.6	104.0 ± 3.7	100.5 ± 2.5	99.1 ± 2.3	101.0 ± 2.8
4 Hr	100.3 ± 2.6	101.2 ± 2.7	85.6 ± 12.2	98.4 ± 2.9	99.2 ± 2.4	100.2 ± 2.9	99.5 ± 2.0
6 Hr	101.1 ± 3.6	---	106.2 ± 4.9	101.0 ± 10.0	100.9 ± 2.5	101.1 ± 1.8	100.0 ± 2.3
8 Hr	102.3 ± 2.6	---	108.8 ± 4.5	94.1 ± 5.8	101.6 ± 1.9	101.2 ± 2.6	101.6 ± 1.7

TABLE 4

RED BLOOD CELL VOLUMES AND COUNTS IN BLOOD COLLECTED IN 6 ANTICOAGULANTS AND STORED AT ROOM TEMPERATURE FOR 8 HOURS (MEAN ± SD, n=9)

	Na_2EDTA	HEPARIN	NaCITRATE	ACD (11.9%)	ACD (15%)	ACD/ Na_2EDTA
Overall Mean RBC Volume (μ^3)	87.0 ± 2.2	86.4 ± 2.3	86.3 ± 2.1	93.6 ± 2.5	91.4 ± 2.5	91.8 ± 2.7
Average Measurement Variation Mean Over the 8-hours (%)	0.45 ± 0.19	0.70 ± 0.30	1.03 ± 0.34	0.74 ± 0.33	0.62 ± 0.34	0.91 ± 1.03
RBC Count ($10^6/\mu l$)	5.21 ± 0.30	5.16 ± 0.40	5.28 ± 0.33	5.09 ± 0.33	5.16 ± 0.28	5.06 ± 0.38
Average Measurement Variation Mean Over the 8-hour (%)	0.57 ± 0.21	0.49 ± 0.17	2.14 ± 0.98	0.48 ± 0.18	0.73 ± 0.47	0.42 ± 0.16

WHITE BLOOD CELL COUNTS IN BLOOD COLLECTED IN 6 ANTICOAGULANTS AND STORED AT ROOM TEMPERATURE FOR 8 HOURS (MEAN ± SD, n=9)

WBC Count (x $10^3/\mu l$)	6.23 ± 0.83	5.31 ± 0.65	6.50 ± 0.73	6.04 ± 0.73	6.02 ± 0.61	6.05 ± 0.77
Average Measurement Variation Mean Over the 8-hours (%)	1.90 ± 1.80	16.9 ± 7.60	5.71 ± 4.59	1.48 ± 0.74	2.82 ± 2.42	2.31 ± 1.80

TABLE 5

A. Relationship of Mean Platelet Volume to Osmolality[1] (Mean ± SD, n=5)

OSMOLALITY mOsm/kg	MPV (μ^3)
270	9.9 ± 0.7
285	9.7 ± 0.6
305	9.5 ± 0.7
315	9.4 ± 0.7
330	9.3 ± 0.7

[1] Blood collected in ACD (15%) and MPV measured in PRP-PBS whose osmolality was preadjusted with water and sodium chloride

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B. Relationship of Mean Platelet Volume to pH[2] (Mean ± SD, n=4)

pH	MPV(μ^3)
6.7	9.6 ± 0.3
6.8	9.7 ± 0.4
6.9	9.7 ± 0.3
7.0	9.6 ± 0.3
7.1	9.6 ± 0.3
7.2	9.6 ± 0.3
7.3	9.6 ± 0.3
7.4	9.6 ± 0.4

[2] Blood collected in ACD (15%) and MPV measured in PRP-PBS whose pH was preadjusted with 0.5 M NaOH.